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44. That the accounts be kept so that the executive officer can determine without delay the cost of operating his department at any time, or the cost of any activity being carried on by his department.

45. That all officials employed in the health department after its reorganization receive at first a probationary appointment only, in order to determine personality and qualifications for public-health work before giving them a permanent position.

46. That the city of Birmingham construct a hospital in which to isolate persons suffering with communicable diseases, with the exception of tuberculosis, such hospital to contain not less than 30 beds.

47. That the county of Jefferson construct, as soon as possible, a sanatorium in which to isolate open cases of tuberculosis. Such hospital to contain not less than 100 beds.

48. That the necessary laws be enacted authorizing the health officer to isolate cases of communicable diseases in hospital when in his opinion it is advisable.

THE LABORATORY DIAGNOSIS OF DIPHTHERIA.

By NEWTON E. WAYSON, Assistant Surgeon, United States Public Health Service.

During the winter months of 1915–16, two diphtheria epidemics, one in West Virginia and one in a reformatory school for boys, in Washington, D. C., together with the consequent school surveys, afforded material for tests of current practice and belief with reference to diphtheria diagnosis.

Three points were studied in some detail, namely:

1. The relation between morphology and virulence for guinea pigs.
2. The relative value of pure and mixed cultures for virulence tests on guinea pigs.
3. The effect on morphologic diagnosis of 2[illegible] hours' interval, at room temperature, between making and incubating the culture, as contrasted with that of immediate incubation.

The Relation Between Morphology and Virulence for Guinea Pigs.

The relation between morphology and virulence for guinea pigs resolves itself practically into the question of the virulence of the solid-staining types of organism. The avirulent granular types occur relatively seldom in the throat and nose, even in convalescents from infection with virulent granular types.

The literature reveals a difference of opinion, based on the tinctorial classification of Wesbrook, among laboratory diagnosticians concerning the clinical virulence and guinea-pig virulence of these solid-staining types, as is shown in the following table:

	A	A_1	A_2	B	B_1	B_2	C	C_1	C_2	D	D_1	D_2	E	E_1	E_2	F	F_2	G	G_2
1. A. P. H. A. (1911)[1]	+	±	......	......	......	......	+	+	......	+	±	......	±	±	......	......	......	±	......
2. Rickards (1908)[2]	+	+	±	......	±	±	+	±	±	+	......	......	±	......	......	......	......	......	......
3. Boston (city) (1900–1903)[3]	+	±	±	......	±	±	+	±	±	+	......	......	±	......	......	......	......	......	......
4. Hill (1907)[4]	+	......	......	......	......	......	+	......	......	+	......	......	......	......	......	......	......	......	......
5. Kolmer (1912)[5]	+	±	±	+	±	±	+	±	±	+	±	±	+	—	—	—	—	—	......
6. Wesbrook (1905)	+	......	......	......	......	......	+	......	......	+	......	......	......	......	......	......	......	......	......
7. Wesbrook (1900)	......	......	......	......	......	......	+	+	......	+	+	......	......	......	......	+	......	......	......
8. Graham-Smith (1913)	+	+	±	+	±	±	+	+	±	+	+	+	+	+	±	±	±	......	......

[1] Opinion of majority. One member includes E_2 and other solid types brilliantly stained.
[2] A, C, D in small numbers are doubtful, often clear up clinically.
[3] Other forms negative or atypical.
[4] A, B, C, D, E significant. A, B, C, D doubtful.
A_2, B_2, C_2, D_2 doubtful: request second culture.
[5] A, C, D virulent. Repetition of examinations in suspicious findings is important.
+ Found pathogenic for guinea pigs.
± Doubtful, or unknown pathogenicity.
— Nonpathogenic for guinea pigs.

Some of the conflicting views are caused by the change, or at least apparent change, from the solid to the granular forms in subculture; and from granular to solid-staining types in direct throat cultures, with the progression of the convalescence of the patient. The following objections have been raised to this alleged change in the staining characteristics: (*a*) That the granular forms are present in the original culture, but are missed in making the smear, or become the predominant type with further growth; and (*b*) that ordinarily the organisms constantly breed true to morphologic type, through several generations on artificial-culture media. However, practically all workers seem to agree on the fact that young cultures are frequently solid staining, later becoming granular, and most of them concede that even *B. hoffmanni* frequently presents granular involution forms in old cultures; further, this organism exhibits considerable pleomorphism in size, thus suggesting that, as a criterion of the virulence of the diphtheria bacillus, morphologic or tinctorial characteristics are uncertain.

The control of the spread of diphtheria is, practically, the control of those persons harboring virulent diphtheria bacilli. The only (approximate) test of virulence practicable in the laboratory is the guinea-pig test.

In this study, staining was done with the standard Löffler's methylene blue, allowing it to act a few seconds, a method which affords a good definition of the granules. Wesbrook's tinctorial classification was used as a guide, and the identification of types of the original culture was, in most instances, checked by a second worker.

Of the cultures from 37 cases tested for virulence during the study, 23 were of the solid-staining varieties of organism, only 3 of which were virulent to guinea pigs. Two of these three which were virulent had, subsequent to the original throat culture, become granular staining in subcultures, and remained granular staining when isolated in pure culture. The third culture remained solid staining and was virulent.

Table showing relation of morphology to virulence, and results, by death or survival of guinea pigs, after inoculation with mixed and pure cultures of B. diphtheriæ.

Culture No.	Type diagnosis.	Mixed culture $\frac{1}{10}$–$\frac{1}{5}$ slant.	Subsequent diagnosis.	Pure cultures.		Remarks.
				1 oese.	1 c. c. 48 hour br.	
2372	D_2	S				
2372+A	D_2	S				
2366	C	D	C	D	D	
2366+A	C	S	C	S	S	
2373	D_2	S				
2373+A	D_2	S				
2410	D_2	S	D_2	S	S	
2410+A	D_2	S	D_2	S	S	
2405	A.C. very few	S				
2405+A	A.C.	S				
2406	D_2	S	D_2	S		
2406+A	D_2	S	D_2	S		
2408	D_2	S	D_2	S	S	
2408+A	D_2	S	D_2	S	S	
2400	C	D				
2400+A	C	S				
2432	D_2	S	D_2	S	S	
2432+A	D_2	S	D_2	S	S	
2413	C	D	C	D		
2413+A	C	S	C	S		
2469	D_2	S				
2469+A	D_2	S				
2448	D_2	S	D_2	S		
2448+A	D_2	S	D_2	S		
2450	D_2	S	D_2	S	S	
2450+A	D_2	S	D_2	S	S	
2456	D_2	S				
2456+A	D_2	S				
2455	D_2	S	D_2	S	S	
2455+A	D_2	S	D_2	S	S	
2524	C	D				
2524+A	C	S				
2510	D_2	S	D_2		S	
2510+A	D_2	S	D_2		S	
2522	D_2	S				
2522+A	D_2	S				
2530	C	D				
2530+A	C	S				
2527	D_2	D				Had been positive case clinically C types.
2527+A	D_2	S				
2521	C	D	C		D	Sore throat (survey).
2521+A	C	S	C		S	
2529	C	S	C		D	Release culture.
2529+A	C	S	C		S	
2525	C	D			D	Bronchi sepsis. Release culture.
2525+A	C	S			DS	
2526	C	D	C		D	Release culture.
2526+A	C	S	C		S	
2505	C	D			D	Sore throat (survey).
2505+A	C	S			S	

Table showing relation of morphology to virulence, and results, by death or survival of guinea pigs, after inoculation with mixed and pure cultures of B. diphtheriæ—**Contd.**

Culture No.	Type diagnosis.	Mixed culture $\frac{1}{10}$–$\frac{1}{5}$ slant.	Subsequent diagnosis.	Pure cultures.		Remarks.
				1 oese.	1 c. c. 48 hour br.	
2523	D_2	S				
2523+A	D_2	S				
2528	A.C.D_2	D				Release culture.
2528+A	D_2	S				
2507	D_2	S				
2507+A	D_2	S				
DC24	D_2	D	C		D	Poor findings. Post-mortem.
DC24+A	D_2	D	C		S	
DC39	C	D	C		D	
DC39+A	C	S	C		S	
DC4	D_2	D	C	D		
DC4+A	D_2	S	C	S		
AP	C	D	C		D	Release culture.
AP+A	C	D	C		S	
AP	C				D	Broncho sepsis. On post-mortem.
AP+A	C				S	
2787	D_2	S	D_2		S	
2787+A	D_2	S	D_2		S	
2857	D_2	S	D_2		S	
2857+A	D_2	S	D_2		S	
2798	D_2	S	D_2		S	
2798+A	D_2	S	D_2		S	
2855	D_2	S	D_2		S	
2855+A	D_2	S	D_2		S	
2557	D_2	S	D_2		S	
2557+A	D_2	S	D_2		S	

Cultures Number +A=control with antitoxin.
Types are indicated according to Wesbrook's classification.
S=survived 10 days or more.
D=dead in less than 7 days.
AP culture killed control in mixed culture in 2 days and unprotected pig in 1 day, both with typical findings.
2525 culture, control dead with pure culture, but in 8 days complicated with an infection with *B. bronchisepticus.*
DC 24 control dead in 1 day, but not with post-mortem findings of diphtheria.

These results conform with the general consensus of opinion, that the solid-staining, Hoffmann-like, or Wesbrook D_2 types, are usually avirulent organisms. The importance of this fact is emphasized by the great frequency with which this type of organism is found in the throats and noses of children other than probable contacts. Further, the exact simulation in form of these organisms by virulent diphtheria bacilli, in many instances, especially in young cultures 5 to 15 hours old, and the apparent change to these types in convalescence, indicate that the virulence test is at present the most reliable means of determining whether the organism in question will produce diphtheria clinically.

Virulence is tested on guinea pigs of 200 to 400 grams weight, and the accepted method is the injection of a pure broth culture of the organism, after 48 hours' growth at 37° C.

The methods used in this study involve the second point to be considered:

The Relative Value of Virulence Tests with Mixed Cultures, as Opposed to Pure Cultures.

This point was suggested by the practice in some diagnostic laboratories of injecting into a guinea pig, as a test of virulence, 24-hour mixed cultures washed from a slant, without using a control.

Twenty-five of the above-mentioned 37 cultures were studied in mixed and pure cultures.

The mixed cultures were incubated 24 hours on Löffler's blood serum mixture, and after the smear diagnosis was made, immediately suspended in 5 cc. of salt solution, of which 0.5 or 1 cc. was injected into each of two guinea pigs, one of which was protected with antitoxin. Or, for the sake of convenience, the culture was allowed to stand at a temperature of 15° C. for from 24 to 96 hours, and then transferred to the serum mixture, incubated for 24 hours, checked microscopically against the original smear findings, and injected into guinea pigs.

The control animals were injected intraperitoneally, or intracardially, with 200 to 350 units of antitoxin, an amount more than adequate for protection, either 24 hours, or 5 hours, previous to the injection of the cultures.

Pure cultures were isolated (*a*) by diluting in broth, carrying over to Löffler's serum mixture, incubating, and fishing colonies from the serum mixture; (*b*) by growing for 24 hours in a serum-water mixture, and, from the film, carrying over to Löffler's serum mixture, incubating, and fishing; or, (*c*) the simplest and seemingly the most efficient method by planting on Löffler's serum mixture from the film of the suspension remaining in the original mixed culture. This suspension should first be allowed to stand at room temperature overnight, a portion of it having been used to test the virulence of the mixed culture.

The cultures were tested for virulence by injecting 1 cc. of a suspension in salt solution, made from one oese of the growth on the Löffler's serum mixture; or, by injecting 1 cc. of a 48-hour, sugar-free, broth culture, into each of two guinea pigs, one of which was protected by antitoxin. The broth cultures were used only after the organism had become accustomed to grow in broth, producing either a good pellicle, a sediment, or, as occurred with several of the solid staining types, a diffuse cloudiness.

The comparison of the results of injecting mixed cultures or pure cultures showed practically no difference in their virulence reaction toward guinea pigs; the post-mortem findings in those dead of the mixed culture corresponding to those found in guinea pigs dead of a known diphtheria toxin. One culture of the 25 tested in pure and mixed culture killed in the former but not in the latter instance, probably owing to the small dose of organisms in the mixed culture, a feature which could be avoided by enrichment.

Four sets of intradermic tests, using four tests to a pig, injecting 0.2 cc. of a suspension in 20 cc. of salt solution, of a 24-hour Löffler's serum mixture slant, showed that the method was not wholly satisfactory. In several instances, using pure cultures, redness and infiltration of the skin were obtained, the cultures subsequently proving avirulent. In one instance a culture producing a very slight skin reaction killed in subcutaneous injection, while the antitoxin control survived. The intensity of a skin reaction, as contrasted with the definite death of an animal, seems an unsafe test for virulence, although, in many instances, it may materially aid diagnosis and shorten the length of time necessary for a positive reaction. The skin reaction occurs in 24 to 48 hours, while the reaction of death of the guinea pig frequently requires from four to seven days. The intradermic reactions, with the use of mixed cultures, are complicated by the pyogenic organisms, ulceration occurring frequently.

The table presented above shows, on the one hand, the relation between types and virulence, and on the other, the virulence relations between mixed and pure cultures.

Morphologic Diagnosis, After a 24-Hour Interval, at Room Temperature, Between Making and Incubating the Culture, and After Immediate Incubation.

Since in the epidemic in West Virginia, by reason of transportation, the cultures examined stood for 24 hours on the media, an experiment was made to test the probable effect on positive diagnosis of this interval previous to incubation.

A set of cultures was made by inoculating Löffler's serum-mixture slants with swabs from 20 throats, allowing them to grow for 24 hours, and examining them carefully under the microscope for diphtheria or diphtheroid organisms. The growth of each tube was then suspended in salt solution, thoroughly mixed, and inoculated heavily on each of two Löffler serum slants, over which was flowed 0.2 cc. of a salt solution suspension of a 24-hour Löffler serum-slant growth of a typical diphtheria bacillus. These suspensions were diluted so as to represent doses of, approximately, 300,000, 30,000, and 300 diphtheria bacilli. One of the slants so treated was incubated at once, the other was left at room temperature for 24 hours and then incubated. Each set of tubes was incubated for, approximately, 17 hours at 37° C. just previous to examination by smear. The smears were made from most of the surface of the slant, and stained by Löffler's methylene blue.

Forty tubes, each with 300,000 diphtheria bacilli inoculation, and 40 with 30,000 were positive, both in the 20 tubes immediately incubated and in those allowed to stand for 24 hours before incubation.

Of 80 tubes inoculated with 300 bacilli, two series of 40 each showed 12 positive and 8 negative of those immediately incubated and 8 positive and 12 negative after 24 hours' standing; a second series showed 19 positive and 1 negative with immediate incubation and 18 positive and 2 negative after standing.

All of the tubes having the 300 organisms showed few organisms to the smear. The controls, made in each lot by inoculating a slant with the diphtheria bacillus only, showed in the first of the 300 series from 18 to 25 colonies, with a small coalescent growth at the bottom of the slant. In making the smears throughout these series, and to approximate more closely the usual swab method of inoculation, which was impracticable in these experiments, the growths at the bottom of the slants were avoided.

Conclusions.

The above findings suggest that the practice of making diagnoses from cultures 24 hours in transportation before incubation at 37° C. was not productive of great error.

The test of virulence by the injection of one-tenth to one-fifth of the 24-hour growth on blood-serum mixture of a mixed culture of the diphtheria bacillus into a guinea pig, controlled by a second injection into a pig protected by antitoxin, was in this series a reliable test. This method may offer a more practicable way of controlling carriers of organisms, morphologically diphtheria bacilli, in that it requires much less manipulation and less time for its execution. The factor of manipulation would be a tremendous saving to the laboratory, and the time element would facilitate quarantine.

The evidence from this study further substantiates the opinion that solid-staining types of the diphtheria bacillus are usually avirulent, and that morphology alone is an insufficient index of the virulence of the diphtheria bacillus.

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